

Genomic cloning and restriction site mapping of a porcine adenovirus isolate: demonstration of genomic stability in porcine adenovirus

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Summary. Restriction endonuclease maps were constructed for the genome of a porcine adenovirus (PAV), NADC-1, which was isolated in 1972 from an adult swine. Genomic DNA libraries of NADC-1 Bam HI, Eco RI/Bam HI, and Sph I fragments were cloned into pUC-18. Using the cloned NADC-1 Bam HI and Eco RI/Bam HI fragments as probes, Southern blot hybridizations were performed to human adenovirus 2 (Ad-2) restriction fragments to determine the left-to-right orientation of the Bam HI and Eco RI/Bam HI fragments. Genomic NADC-1 DNA was cleaved with ten restriction endonucleases (RE). Using cloned NADC-1 genomic fragments as probes in Southern blot hybridizations, an RE site map was constructed. Nucleotide sequencing of four clones confirmed several RE sites. The size of the NADC-1 genome was determined to be approximately 32 kbp. The size of Hind III, Xba I, Sma I, Eco RI, Bam HI, Bgl II, Pst I, and Sph I RE fragments from NADC-1 was compared to those from the reference strain of PAV serotype 4 (F 618), and to two recent isolates, NADC-2 and NADC-3. For all restriction enzymes examined, the sizes of the NADC-1 fragments were identical to PAV-4, NADC-2, and NADC-3 fragments, indicating that the NADC-1 isolate is very closely related, if not identical, to PAV-4 and two recent isolates. Southern blot hybridizations also indicated that NADC-1, NADC-2, NADC-3, and PAV-4 are very similar and revealed regions of sequence similarity between NADC-1 and human Ad-2 and human Ad-5.

Introduction

Adenoviruses are double-stranded DNA viruses that have been isolated from a large number of animal species, including swine. Numerous members of the *Adenoviridae* family have been described. Although there is broad antigenic

cross-reactivity between the complement-fixation antigen within each genus, there is no common antigen that characterizes the entire family [13]. Adenoviruses have been used as recombinant viral vaccine vectors for protection against several pathogenic viruses, such as hepatitis B virus, human immunodeficiency virus type 1, pseudorabies virus, and rabies virus (for review see [8]). Although different adenovirus serotypes have been employed in these studies, human Ad-5 has been used most extensively. Heterologous DNA sequences have been inserted into several regions of the genome, but the early transcription region 3 (E 3) may be most useful since it appears to be dispensable for replication *in vitro*.

There are four distinguishable serotypes of porcine adenovirus (PAV), designated 1 through 4. Porcine adenovirus was first isolated from a rectal swab of a piglet with diarrhea [9] and subsequently from brain tissue of a pig with encephalitis [14]. While the majority of infections cause no disease, PAV has been associated with encephalitis, pneumonia, kidney lesions, and diarrhea [4]. Adenovirus infection in swine may be common. Using an indirect fluorescent antibody test, Dea and El Azhary [3] found 15% of sera from 540 adult pigs with respiratory disease to be seropositive for PAV-4. Strains of PAV-4 appear to be the most widespread [4]. Restriction endonuclease (RE) digestion patterns have been described for PAV serotypes 1, 2, and 3 using the enzymes Bam HI, Eco RI, Hind III, and Pae R7 [7]. These investigators reported that PAV serotypes 1–3 were closely related, although distinguishable, at the genomic level and that none of the RE patterns resembled those from human, murine, canine, bovine, or fowl adenovirus. Benkö et al. [1] demonstrated that genomic human adenovirus serotype 2 (Ad-2) probes hybridized to PAV-3 in Southern blots under low-stringency conditions, indicating nucleotide sequence similarity. If PAV is to be considered for use as a recombinant viral vaccine vector, a more detailed study of the molecular and *in vitro* characteristics is necessary.

We describe the molecular cloning and RE site mapping of a PAV, NADC-1, isolated in the early 1970's. Restriction endonuclease cleavage patterns of NADC-1 were compared to those of the reference strain for PAV-4, which was isolated in 1966 by Kasza [14], and two additional PAV (NADC-2 and NADC-3), isolated from rectal swabs of young pigs in 1992. Southern blot hybridizations using high stringency conditions were performed to determine the level of sequence similarity. Based on these experiments, it appears that the three isolates are likely to be strains of PAV-4 and that the genome of this PAV serotype is remarkably stable in its host. Southern blot hybridizations of genomic NADC-1 DNA to human Ad-2 and human Ad-5 DNA were performed to demonstrate the degree of similarity at the nucleotide level that NADC-1 shares with these two well-characterized adenoviruses.

Materials and methods

Cells and viruses

Primary porcine kidney (PK) cell cultures were prepared from trimmed cortical tissue of mid- to late-term fetuses collected at a local abattoir by standard methodology [6]. Cells

were grown and maintained in Eagle's minimal essential medium (MEM) F-15 supplemented with 0.25% lactalbumin hydrolysate, 10% heat-inactivated fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, and 50 µg/ml gentamicin sulfate at 37°C, 5% CO₂, in a humidified incubator. After either the first or second passage, cells which were 50% confluent were infected with PAV at a multiplicity of infection of approximately 1.0. When 75% of cells were showing cytopathic effect (typically 6 to 8 days postinfection), viral DNA was prepared as described below. The reference strain of PAV-4 (F 618), and human Ad-5 were obtained from American Type Tissue Collection. Human Ad-5 was replicated on human 293 cells using the same media and conditions outlined above. Cytopathic effect in 293 cells infected with human Ad-5 was typically observed 2 days postinfection.

The PAV NADC-1 was isolated from a tonsillar swab of an adult sow in 1972. The PAV isolates NADC-2 and NADC-3 were isolated from rectal swabs of approximately 12-week-old pigs in 1992. Rectal swabs were obtained from a single group of 22 healthy pigs and stored frozen in 4 ml Eagle's MEM F-15 cell culture media (see above) for 2 days. After thawing, the media was filtered through a 0.45 µm filter (Millipore) and inoculated onto PK cells in a 24-well plate. Cell culture supernatant from the inoculated wells containing cells showing cytopathic effect after approximately 7 days was screened with a fluorescein-conjugated antiserum that reacts positively to NADC-1. Two of the samples which caused cytopathic effect (characterized by rounding and detachment of cells) reacted positively with this antiserum and the viruses purified from these samples were designated NADC-2 and NADC-3.

Purification and preparation of viral DNA

Genomic PAV and human Ad-5 DNA were purified by a modification of the Hirt method [12] as described by Shinagawa et al. [20]. Mock DNA purification from cells to which no virus had been added was performed to insure that DNA did not come from PAV already present in PK cells used for virus replication. The left terminal Bam HI fragment of NADC-1 was cloned into pUC-18 by removing the 5' terminal protein with 0.3 N NaOH [11]. Eco RI adaptors were ligated to the blunt ends with T4 DNA ligase (Gibco BRL) following the supplier's instructions. The right terminal Bam HI fragment of NADC-1 was not cloned. Genomic human Ad-2 DNA was purchased from Gibco-BRL.

Cloning of NADC-1 genomic fragments

NADC-1 genomic fragments were cloned into the plasmid vector pUC-18 [25] by standard methodology [17]. Genomic NADC-1 DNA was digested with either Bam HI, Eco RI and Bam HI, or Sph I and ligated to similarly prepared pUC-18 with T4 DNA ligase (Gibco BRL). Competent *E. coli* cells (strain JM 109) were prepared and transformed with recombinant plasmids [11]. Luria-Bertani plates with 100 µg/ml ampicillin, 230 µg/100 mm plate isopropyl β-D-thiogalactopyranoside (IPTG), and 1 mg/100 mm plate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were used to visually screen for recombinants. Plasmid DNA minipreps [2] were subjected to RE analysis to confirm the presence of inserts. Large amounts of selected recombinant plasmids were prepared by Qiagen column purification.

Restriction enzyme analysis

All RE enzymes were supplied by Gibco-BRL and used according to the supplier's instructions. Genomic RE fragments were separated in 0.7 or 1.2% agarose (SeaKem LE, FMC) gels using TAE buffer (40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.4) which contained 0.5 µg/ml ethidium bromide. Molecular size standards used in each gel were bacteriophage lambda Hind III fragments (Gibco BRL) and a 1 kb ladder (Gibco BRL),

shown in Figs. 1 and 4A as lanes M1 and M2, respectively. Following electrophoresis, genomic RE fragments were blotted to Hybond-N (Amersham) by capillary action [17, 21] using 2M ammonium acetate. Blots were baked at 80 °C for 2 h under vacuum prior to hybridization experiments.

Southern blot hybridization analysis

An enhanced chemiluminescent system (ECL direct nucleic acid labeling and detection system catalog number RPN.3000, Amersham) with Kodak X-OMAT AR radiographic film was used for detection following Southern blot hybridizations. For all blots, 0.5 M sodium chloride was added to the prehybridization/hybridization buffer supplied with the system. Blots were allowed to prehybridize for 30 min at 42 °C prior to addition of the probe. Hybridizations were carried out at 42 °C for approximately 16 h. Probes were prepared by first digesting recombinant plasmids with the appropriate RE, then separating the PAV fragment from pUC-18 by electrophoresis in 0.8% low-melting temperature agarose (SeaPlaque, FMC) in TAE buffer. The appropriate bands were cut from the gel and used as probes with no further purification [22]. Labeling of probes was carried out according to supplier's instructions. A single blot was used for numerous hybridizations; following hybridization and detection with one cloned fragment, the blot was stripped with boiling $0.1 \times$ SSC ($20 \times$ SSC is 0.3 M sodium citrate, 3 M sodium chloride, pH 7.0), allowed to cool to room temperature, then reprobed with the next cloned fragment.

Preliminary nucleotide sequence analysis

Double-stranded DNA sequencing [23] with Taq polymerase and fluorescently labeled dideoxynucleotides (Applied Biosystems International, Prism system) [18] was performed

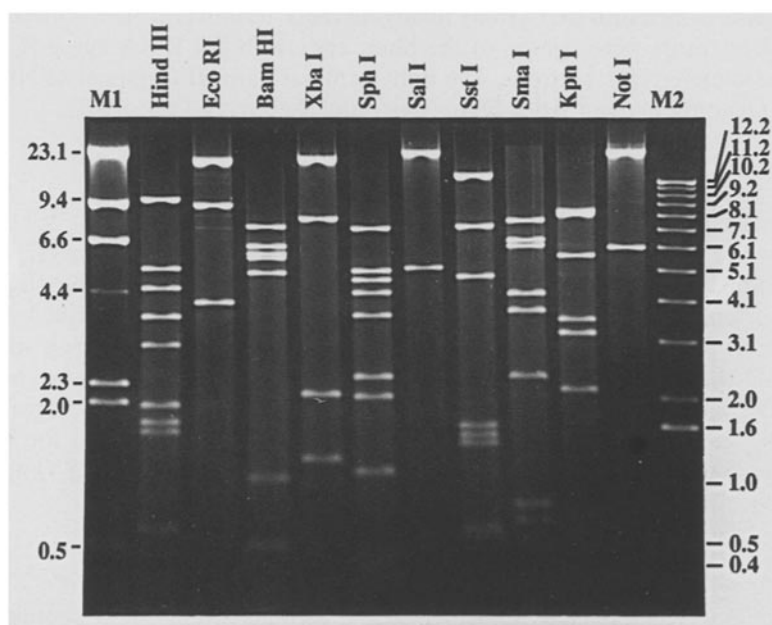


Fig. 1. Agarose gel electrophoresis of PAV NADC-1 RE fragments. Genomic PAV NADC-1 DNA was digested with the indicated RE, separated on a 0.7% agarose gel, and visualized by ethidium bromide staining

for analysis with an Applied Biosystems 373 A automated sequencer. Universal forward and universal reverse primers, along with oligonucleotide primers synthesized from generated sequence, were used in sequencing reactions. A minimum of 0.5 kb of nucleotide sequence was determined for the Bam HI E and F fragments and the Eco RI/Bam HI fragments corresponding to map positions 24.8–28.6 and 81.8–87.3. Computer analysis of sequence information was performed using DNASTAR and Genesis software.

Results

Genomic DNA from a PAV isolate, NADC-1, was digested with the restriction endonucleases (RE) Hind III, Eco RI, Bam HI, Xba I, Sph I, Sal I, Sst I, Sma I, Kpn I, and Not I. The pattern of RE fragments is illustrated in Fig. 1. Digestion of genomic PAV with Hind III, Bam HI, Sph I, Sst I, Sma I, or Kpn I produced fragments ranging in size from 0.4 kbp to 13.5 kbp. Cleavage with Eco RI and Xba I produced three and four fragments, respectively. Cleavage with Sal I or Not I produced two fragments, indicating a single restriction site for each enzyme. The size of each restriction fragment was determined by comparison to known size standards and is listed in Table 1. The size of the NADC-1 genome was found by addition of the RE fragment sizes produced by cleavage with Hind III, Bam HI, Sph I, Sst I, Sma I, or Kpn I and was estimated to be 32 kbp.

The sizes of Bam HI fragments, Eco RI/Bam HI double digestion fragments, and Sph I fragments range from 0.4 to 7.6 kbp, a size range optimal for cloning the PAV genome into a plasmid vector. All of the Bam HI fragments were cloned except for the Bam HI C fragment, which is the right terminal Bam HI fragment. Eco RI/Bam HI double digestion fragments corresponding to map

Table 1. Size of PAV NADC-1 restriction fragment

Fragment	Restriction endonuclease									
	Hind III	Eco RI	Bam HI	Xba I	Sph I	Sal I	Sst I	Sma I	Kpn I	Not I
A	9.6	18.9	7.5	20.8	7.4	27	13.5	7.9	8.5	26.1
B	5.2	9.2	6.3	7.9	5.1	5.2	7.5	6.5	8.3	6.1
C	4.5	4.1	5.8	2.1	4.8		5.0	6.3	5.7	
D	3.6		5.7	1.4	4.2		1.7	4.4	3.6	
E	3.0		5.0		3.8		1.6	3.8	3.2	
F	2.0		1.1		2.4		1.5	2.4	2.2	
G	1.7		0.5		2.1		0.7	0.9	0.8	
H	1.6				1.2		0.5	0.7		
I	0.7				0.5					
J					0.4					
Sum	31.9		31.9		31.8		32	32.9	32.3	

The length (in kbp) of DNA fragments was determined from measurements of the relative mobilities of the fragments in 0.7% and 1.2% agarose gels. The sum of fragment sizes was determined where the largest fragment was ≤ 13.5 kbp

positions 24.8–28.6, 28.6–42.6, and 81.8–87.3 were cloned. All Sph I fragments were cloned except for the Sph I F and H fragments, which are the two terminal Sph I fragments.

To assemble the RE site maps (Fig. 2), each cloned Bam HI and Eco RI/Bam HI fragment was hybridized first to the Bam HI, Eco RI, and Sph I restriction fragments of human Ad-2. The patterns of hybridization gave the left-to-right orientation of these NADC-1 fragments (data not shown). The location of four of the cloned NADC-1 fragments (the Bam HI E and F fragments and the Eco RI/Bam HI fragments corresponding to map positions 24.8–28.6 and 81.8–87.3) was confirmed by nucleotide sequencing followed by a computer

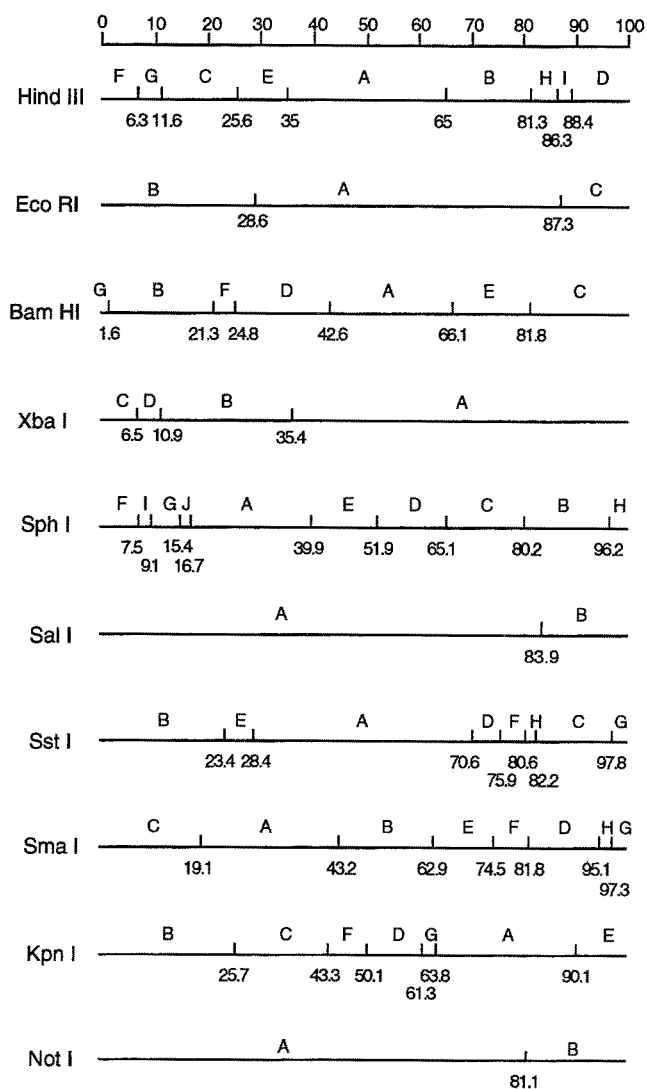


Fig. 2. Location of the cleavage sites for ten RE enzymes in PAV NADC-1 DNA. The location of RE cleavage sites is given in map units. Each map unit represents 320 bp

search of the GenBank data files for nucleotide sequence similarity to human Ad-2 and other adenoviruses. The nucleotide sequence of the cloned NADC-1 fragments was typically 60–70% similar over ≥ 300 bases to human Ad-2. For each fragment, the sequence similarity was in the same region predicted by Southern blot hybridizations.

Each cloned Bam HI and Eco RI/Bam HI fragment, plus the Sph I E and J fragments, was sequentially hybridized to a blot prepared from the genomic NADC-1 RE fragments represented in Fig. 1. Analysis of the hybridization patterns allowed construction of the RE site maps shown in Fig. 2. One map unit represents approximately 320 bp for NADC-1. Two representative Southern blot hybridizations to NADC-1 RE fragments are presented in Fig. 3. In Fig. 3 A, the blot was probed with the Eco RI/Bam HI fragment corresponding to map position 81.8–87.3. In Fig. 3 B, the blot was probed with the Bam HI G fragment, the left-terminal Bam HI fragment. Cleavage of two cloned fragments with a second RE was used to define the location of three sites. To confirm the location of the Sph I G and I fragments, the Sph I G fragment was cleaved with Hind III. To confirm the position of the Kpn I D, F, and G fragments, the Sph I E fragment was cleaved with Kpn I. Sixteen of the 50 RE sites mapped (Fig. 2) have been verified by nucleotide sequencing of the Bam HI

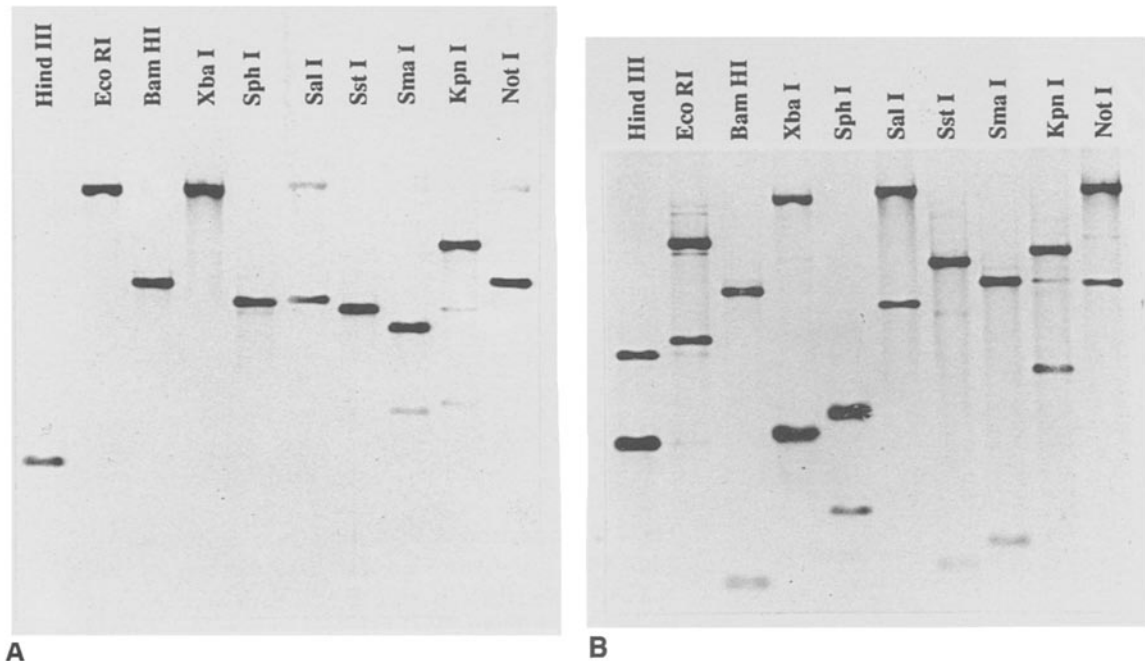


Fig. 3. Southern blot hybridization analysis of PAV NADC-1 RE fragments. **A** Southern blot hybridization of the fragments shown in Fig. 1 probed with the Eco RI/Bam HI fragment that corresponds to map position 81.8–87.3. **B** Southern blot hybridization of the fragments shown in Fig. 1 probed with the Bam HI G fragment, which is the left terminal Bam HI fragment

E and F fragments and the Eco RI/Bam HI fragments corresponding to map positions 24.8–28.6 and 81.8–87.3 (data not shown).

The Sph I RE fragment patterns of NADC-1, NADC-2, NADC-3, and the reference strain for PAV-4 are shown in Fig. 4 A, lanes 1–4. For Sph I, as well as Hind III, Xba I, Sma I, Eco RI, Bam HI, Bgl II, and Pst I (data not shown), little or no variability in RE pattern existed among the different PAV isolates.

The RE fragment patterns of the three PAV isolates, and PAV-4, human Ad-2, and human Ad-5 for Sph I (Fig. 4 A) were also compared. Figures 4 B

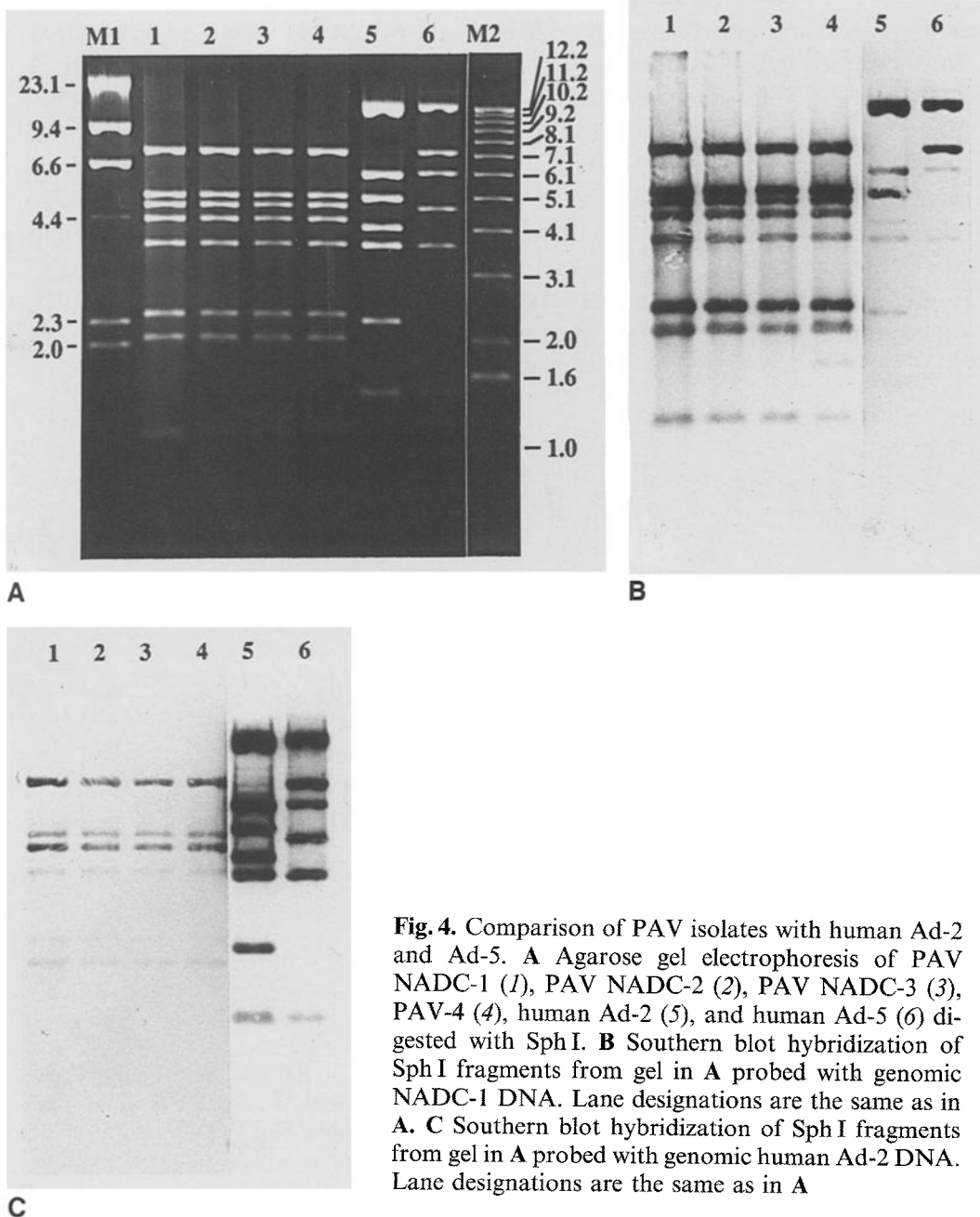


Fig. 4. Comparison of PAV isolates with human Ad-2 and Ad-5. **A** Agarose gel electrophoresis of PAV NADC-1 (1), PAV NADC-2 (2), PAV NADC-3 (3), PAV-4 (4), human Ad-2 (5), and human Ad-5 (6) digested with Sph I. **B** Southern blot hybridization of Sph I fragments from gel in A probed with genomic NADC-1 DNA. Lane designations are the same as in A. **C** Southern blot hybridization of Sph I fragments from gel in A probed with genomic human Ad-2 DNA. Lane designations are the same as in A.

and C represent Southern blot hybridizations of the gel in panel A probed with genomic NADC-1 DNA and human Ad-2 DNA, respectively. For the blot probed with genomic NADC-1, all bands from NADC-2, NADC-3, and PAV-4 were recognized after a short exposure of the radiographic film. The presence of an additional band in the digestion of PAV-4 DNA most likely represents an artifact caused by addition of excess enzyme. All of the bands from human Ad-2 and human Ad-5 were recognized when the same blot was exposed to the radiographic film for a much longer time, although the Sph I D and G fragments of human Ad-2 and the Sph I D and F fragments of human Ad-5 were recognized less strongly than other fragments. For human Ad-2, these bands correspond to the left end of the E2B region and E4 region, respectively. When the blot was probed with genomic human Ad-2, all bands from human Ad-2 and Ad-5 were recognized after a short exposure of the radiographic film, but three bands, the Sph I B, E, and F fragments, from all PAV isolates were recognized only weakly when the same blot was exposed to the radiographic film for a much longer time. Stringency conditions were the same for all Southern blot hybridizations.

Discussion

A PAV isolate, NADC-1, has been cloned and subsequently mapped using ten RE enzymes. The size of the NADC-1 genome was estimated to be 32 kbp, a value that is within the range of most other members of the *Adenoviridae* family. Benkö et al. [1] used Southern blot hybridization analysis to demonstrate that PAV-3 had nucleotide sequence similarity to human Ad-2. Since it has been reported that the genome organization of canine [5], bovine [14], and equine [19] adenoviruses is similar to human Ad-2, the orientation of cloned NADC-1 genomic Bam HI and Eco RI/Bam HI fragments was determined by Southern blot hybridization to human Ad-2 restriction fragments. Once this was known, the location of other RE fragments was determined by Southern blot hybridization of cloned NADC-1 genomic Bam HI, Eco RI/Bam HI, and Sph I fragments to NADC-1 RE fragments. The location of several RE fragments was confirmed by preliminary nucleotide sequencing and cleavage of cloned fragments with a second RE. Approximately 96% of the NADC-1 genome was cloned, including the left terminal Bam HI fragment. The only region of the genome that was not cloned is the right terminal 1.2 kbp. Difficulty in cloning the terminal fragments was presumably due to the presence of a terminal protein, which is covalently linked to the 5' end of each strand [16].

The positions of two Bam HI and two Eco RI/Bam HI RE fragments on the map were confirmed by nucleotide sequencing of the cloned fragments (data not shown). The sequence data generated were aligned with human Ad-2 sequences, and in each case, the cloned NADC-1 fragments had homology to the same region predicted by Southern blot hybridization analysis. Therefore, as reported for adenoviruses isolated from other species, the genome organization of NADC-1 appears to be similar to human Ad-2. Nucleotide sequence similarity between NADC-1 and human Ad-2 was typically 60 to 70% over ≥ 300 bases

in areas of the genome representing the left end of the E2B region of human Ad-2, and domains of the L4 and L5 regions coding for the pVIII and fiber genes of human Ad-2, respectively [24]. However, some NADC-1 nucleotide sequences in these same regions, plus all of the putative E3 region examined, had considerably lower levels (30–35%) of sequence similarity to human Ad-2. These findings were corroborated by Southern blot hybridization experiments presented in Figs. 4B and C. These data indicate that NADC-1 has similarity at the nucleotide level throughout the genome with human Ad-2 and Ad-5. However, since several restriction fragments of human Ad-2 and Ad-5 were recognized more strongly by genomic NADC-1 than others, it would appear that some regions of the genome are more highly conserved than others.

Many adenoviruses have an inverted terminal repeat of 100–140 bases long [13] and this also appears to be true for NADC-1. It was demonstrated that for each RE digestion, two bands from each lane were recognized by the left-terminal Bam HI fragment (Fig. 3B), a result which did not occur when the blot was probed with any other cloned fragment. In each lane one fragment was recognized more strongly, which would suggest that this was the left terminal fragment, while the other was the right terminal fragment.

Based on RE patterns and Southern blot hybridization studies, it appears that NADC-1, isolated in 1972, is very similar to the reference strain of PAV-4, isolated in 1966 [14]. Even if the additional Sph I site in PAV-4 is not an artifact, the band at 1.7 kbp is recognized by genomic NADC-1 (Fig. 4B), indicating that a similar region is present in the genome of both viruses. Two isolates made in 1992, NADC-2, and NADC-3, had identical restriction patterns to NADC-1 for several enzymes. These findings demonstrate that strains of what is likely to be PAV-4 are very stable in the adapted host. Restriction patterns of NADC-1 for the enzymes Eco RI, Bam HI, and Hind III are quite different from those of PAV serotypes 1–3 for the same enzymes [7].

In conclusion, we have reported the first detailed molecular characterization of a porcine adenovirus and, with the genomic clones of NADC-1, provided a basis for future studies. Southern blot hybridizations and preliminary nucleotide sequence analysis indicated that the genome organization of NADC-1 is similar to that of human Ad-2 and other adenoviruses. The presence of inverted terminal repeats in NADC-1, as in other adenoviruses, was implied by these studies. We have also demonstrated that the genome of NADC-1 is very stable. This characteristic makes NADC-1 a candidate for use as a viral vaccine vector, although the similarity we have demonstrated between NADC-1 and PAV-4 may be a limitation to the use of NADC-1 in this role since PAV-4 may be widespread in the swine population.

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